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14. ABSTRACT (200 words)
Activation of glucocorticoid receptor signaling and its subsequent alterations has been implicated in the pathophysiological fear response to stress and the pathogenesis of stress-associated psychiatric disorders such as posttraumatic stress disorder (PTSD). To further examine the association between alterations in central glucocorticoid receptor signaling and the occurrence of stress-induced psychiatric symptoms, the present study, utilizing a learned helplessness stress model in rats, will examine whether glucocorticoid receptor signaling activation shortly before or after stress might prevent the occurrence of stress-induced physical and behavioral abnormalities such as exaggerated acoustic startle response (ASR) and reduced body weight gain. Rats subjected to restraint/tail shock for three days have been shown to develop a long-lasting elevated ASR and reduced body weight gain, compared to non-stressed control animals. In this study, the glucocorticoid receptor agonist, corticosterone (3 mg/kg), was administered 30 min before or after exposure of the animals to the stress protocol. Prophylactic administration of corticosterone (3 mg/kg) before stress attenuated the exaggerated acoustic startle response to stress and normalized body weight gain resulting from stress. Current results demonstrate a promising clinical implication for preventing the pathogenesis process of PTSD by corticostrone administration.

15. SUBJECT TERMS
Corticosterone, Rat, PTSD, Stress, Forgetting, Fear, Acoustic Startle

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INTRODUCTION

Subject: It has been observed in normal subjects that forgetting is a spontaneous and/or gradual process in which previous memories are no longer readily retrievable from brain memory storage. The rate of forgetting is generally related to the function of time associated with an event initially experienced (Wixted 235-69). However, in PTSD patients, vivid flashbacks of traumatic memories occur even years after the traumatic incident, indicating that there is a failure of the forgetting process in the PTSD brain. Is there any way to promote the forgetting process in a PTSD subject? Emerging evidence suggests that systemic administration of corticosterone can enhance the memory forgetting process in animals and in healthy humans (de Quervain, Roozendaal, and McGaugh 787-90;Aerni et al. 1488-90). However, whether administration of corticosterone could also reduce fear memory flashbacks in PTSD patients and/or reduce the enhanced acoustic startle reflex observed in an animal model of PTSD, is currently unknown. We hypothesize that administration of corticosterone prior to or shortly after a period of intense, repeated stress will promote the traumatic memory forgetting process and/or prevent traumatic memory retrieval in our animal model of PTSD.

Purpose: Our immediate objective is to use an animal model of PTSD developed in our laboratory to investigate the effectiveness of administration of corticosterone prior to or shortly after a period of intense, repeated stress in promoting the traumatic memory forgetting process and preventing fear memory retrieval. The use of a pharmacologic intervention to promote the memory forgetting process requires that it be introduced in an appropriate time window and in an optimized dose in order to obtain maximum efficacy without inducing adverse side effects. The experiments proposed will measure changes in ASR in stressed and control animals before and after administration of corticosterone. In addition, some animals will receive a corticosterone receptor (CR) antagonist to determine whether the action of corticosterone can be completely blocked.

Scope:

- Innovation: This study will provide information about the cellular mechanisms underlying PTSD and fill a knowledge gap in the current research on PTSD. Such knowledge may facilitate the development of novel pharmacological interventions for the treatment of PTSD.
- Intervention: Administration of novel pharmacologic interventions may help to prevent as well as treat PTSD. More specific interventions provide treatment while minimizing side effects.
- Application: Novel pharmacologic interventions have the potential for broad use both in military populations (those on active duty, reservists and veterans) and in civilian populations exposed to traumatic stress (natural disasters, vehicle crashes, etc.).

BODY

Task 1: Animal Protocol Approvals

Announcement of Concept Award PT073670 to He Li, M.D., Ph.D.(PI) and Lei Zhang, M.D. and Robert J. Ursano, M.D. (CoPIs), was made on January 3, 2008. The Certificate of Environmental Compliance for this project was awarded by the USUHS Environmental Compliance Officer on January 15, 2008 and the PI Assurance Document was signed January 15, 2008. An animal protocol was submitted to the USUHS IACUC on February 8, 2008 and approved on April 24, 2008. A copy of the IACUC approved animal protocol was submitted to the USAMRMC ACURO for review; ACURO approval was issued May 12, 2008. All animal experiments have been done in accordance with IACUC and ACURO approved protocols. A total of 272 animals have been used for the experiments completed thus far.

Task 2: Administration of the Stress Protocol and ASR Measurements

The stress protocol was administered to young adult male Sprague Dawley rats weighing ~150 gm on the first day of stress. Animals were housed 2 to a cage and allowed to acclimate to the USUHS Animal Facility for at least 5 days. No animals died or were injured as a result of exposure to the stress protocol. Stress exposure consisted of a 2-h per day session of immobilization and tail-shocks for three consecutive days. We stressed animals in the morning (between 0800 and 1200). They were restrained in a plexiglas tube, and 40 electric shocks (1 mA, 3 s duration) were applied at varying intervals (140–180 s). This stress protocol was adapted from the ‘learned helplessness’ paradigm in which animals underwent an aversive experience under conditions in which they could not perform any adaptive response (Seligman and Maier 1-9;Seligman and Beagley 534-

41). The rats were stressed for three consecutive days because it had been previously demonstrated that repeated stress sessions for three days were more effective than a single stress session in producing physiological and behavioral abnormalities associated with depression and anxiety disorders (Ottenweller et al. 829-41;Servatius, Ottenweller, and Natelson 539-46). Additional stress sessions do not appear to produce greater physiological and behavioral changes (Ottenweller et al. 689-98;Ottenweller et al. 829-41).

The acoustic startle reflex test was conducted with a Startle Response Acoustic Test System (Coulbourn Instruments, Columbus, Ohio, USA). The system consisted of four weight sensitive platforms in a sound-attenuated chamber, though only one platform was used at a time. A subject's movements in response to stimuli were transduced into analog signals by a piezoelectric unit attached to the platforms. These signals were then digitized and stored by a computer. All acoustic stimuli were given by a loudspeaker mounted 24 cm above the test cage. To minimize the effects of handling and stress on drug testing, animals were allowed to acclimate to the startle chamber for 3 days before experiments. The startle baselines were then tested for each animal on Day -1 before they experienced the stress protocol. During testing, rats were individually placed in an animal holder (E05-15, Coulbourn Instruments, Columbus, Ohio, USA), which was then placed on a weight-sensitive platform within the chamber. A ventilating fan built into the chamber provided background noise. Following a 3-min acclimation period, animals were exposed to six types of stimulus trials: 100 and 110 dB alone, both 100 and 110 dB with pre-pulse (84dB), pre-pulse alone and no stimulus. Each trial type was presented eight times. Trial types were presented in random order to avoid order effects and habituation. Inter-trial intervals ranged randomly from 15 to 25s. Animals were tested in the dark cycle; during the experiments the red light was provided in the dark room. ASR testing of all animals took place on Day 10, 21 and 30 following completion of the stress protocol.

Each animal's responses were averaged within trial type. Trials during which no stimuli were presented were used to control for normal subject movements on the platform. Amplitudes of each trial type were derived by subtracting grams (g) of platform displacement on the no-stimulus trials from g of platform displacement in response to specific stimuli. The remainder resulting from this calculation represented absolute amplitude of ASR to the stimulus (e.g., 100 dB, 100 dB with pre-pulse, 110 dB, 110 dB with pre-pulse). The ASR amplitude tested each time was finally represented as "% of baseline", which was calculated using the equation: % of baseline= (absolute amplitude /baseline absolute amplitude) ×100%. For each test day, ANOVAs for repeated measures were performed on startle amplitudes with factors of stress status and drug dosage. Tukey or Bonferroni test was used to assess significant post-hoc differences between individual groups. The data were represented as mean ± S.E.M

Task 3: Administration of corticosterone pre-stress to determine its effect on acoustic startle response (ASR) was administered to stressed and control animals thirty minutes prior to administration of the stress protocol on three consecutive days. Baseline acoustic startle measurements and plasma levels (obtained from tail blood samples) for stressed and control animals were determined on Day -1 preceding exposure to the stress protocol. Baseline measurements of body weight were made on Day -1 preceding stress both as a physiological measure and as a metric for balancing the groups. After the third and final exposure to stress, animals were weighed immediately after stress and again on Days 1, 7 14, 21 and 28 poststress. For each test day, ANOVAs for measures were performed on net weight gain and average growth rate with the factors of stress status and drug dosage. Tukey or Bonferroni test was used to assess significant post-hoc differences between individual groups. The data were represented as mean±S.E.M.

Task 4: Administration of post-stress to determine its effect on ASR was administered to stressed and control animals thirty minutes after administration of the stress protocol on three consecutive days. Baseline acoustic startle measurements and plasma levels (obtained from tail blood samples) for stressed and control animals were determined on Day -1 preceding exposure to the stress protocol. Body weight measurements were also recorded. The same measurements were made on Days 7, 14 and 21 following completion of the stress protocol. Additional experiments involving post-stress administration of have indicated that post-stress treatment with corticorsterone has less efficacy (no statistically significant difference between stressed group and post-stress

treated group) in attenuating the exaggerated acoustic startle response in this animal model of PTSD Statistical analysis of the data obtained was performed as described above.
Experimental Results of Task 3 and Task 4:

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1. The effect of administration of corticosterone prior to or immediately following stress on stress-enhanced ASR.

We first examined the effect of the stress protocol and administration of the drug (3 mg/kg conrticosterone) or vehicle on ASR. Consistent with previous reports, exposure to three-day restraint/tail shock increased the ASR amplitude on Day 14 following stress $p<0.04$ (Figure 1). The vehicle injection did not produce enough intensity of stress to significantly enhance startle in comparison to non- stressed control animals. The effect of stress on ASR continued to be observed on Day 21 following stress (Figure 2), indicating that the enhanced startle response was a long-lasting effect of this stress protocol. The corticosterone administration was without effect on ASR amplitude similar to that in control animals (on day 14, $p>0.15$). Data analysis further revealed that, in animals exposed to stress, a significantly lower ASR to acoustic startle stimuli was observed one day after stress as compared to vehicle-treated animals ($p <0.02$). However, ASR was enhanced on day 14 after the cessation of stress ($p< 0.04$) (figure 1). It was noteworthy that corticosterone injected immediately following stress was less effective than the administration of this compound prior to stress in decreasing the stress-induced enhancement of ASR (Figure 1).

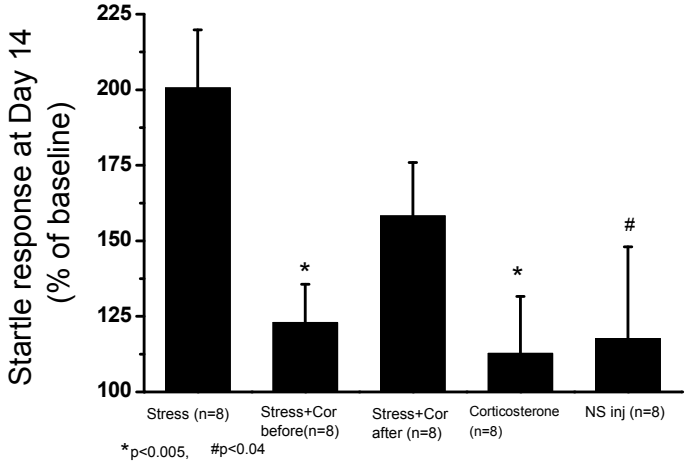

Figure 1. Mean ± S.E.M. of peak startle amplitude (represented as % of the baseline of ASR). Corticosterone 3 mg/kg pretreatment, 3 mg/kg posttreatment and corticosterone alone, stressed and non-stressed controls respectively. The ASR to 100 dB was tested on Day 14 after cessation of the stress protocol.

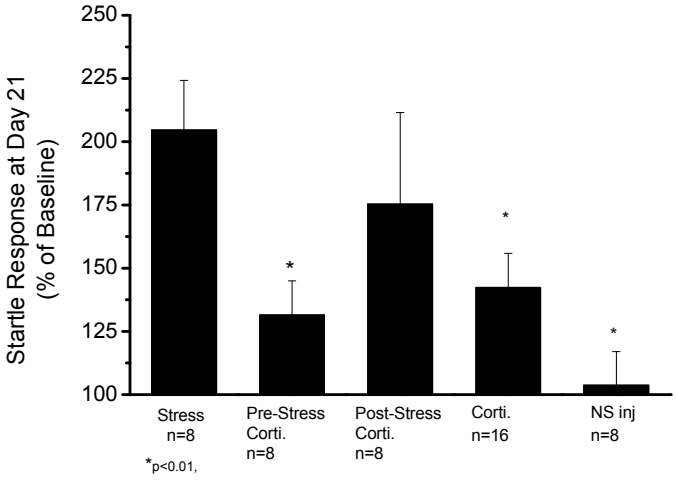

Figure 2. Mean ± S.E.M. of peak startle amplitude (represented as % of the baseline of ASR). Corticosterone 3 mg/kg pretreatment, 3 mg/kg posttreatment and corticosterone alone, stressed and non-stressed controls respectively. The ASR to 100 dB was tested on Day 21 after cessation after stress protocol.

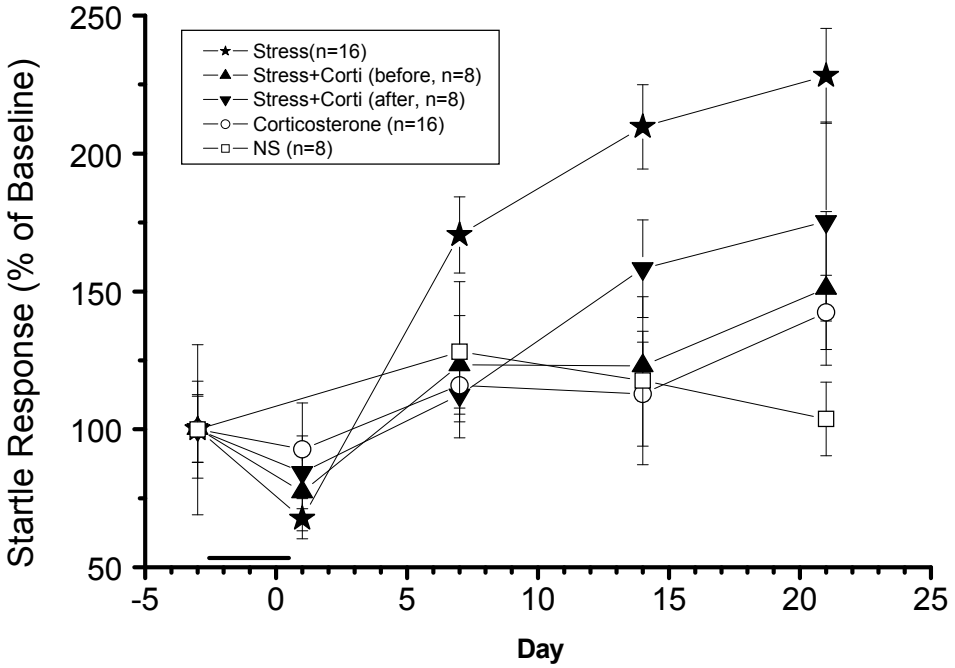

Figure 3. Pretreatment of corticorstrone significantly attenuated exaggerated acoustic startle response induced by stress protocol. Mean ± S.E.M. of peak startle amplitude (represented as % of the baseline of ASR). Corticosterone 3 mg/kg pretreatment, 3 mg/kg posttreatment and corticosterone alone, stressed and non-stressed controls respectively. The ASR to 100 dB was tested on Day 1, and day 7, day 14 and day 21.

2. Administration of corticosterone prior to stress normalized body weight of the stressed animals.

The subjects we used in this study were young adult rats (125-150g) when the experiments began. At this stage, the animals' body weight increased very quickly on a daily basis (5-7 g/day). We thus used the weight gain to assess the differences in body weight across groups. Given that the baseline body weight was balanced across the groups, the differences in body weight gain across groups were actually the differences in body weight across groups. It is a more sensitive way to assess the differences in body weight across the groups due to low variability.

As illustrated by Figure 4, exposure to three-day inescapable tail-shock significantly decreased the body weight gain in stressed animals ($p<0.01$), resulting in a lower body weight than that of their control counterparts. This effect remained as long as 21 days following stress (Figure 5). Corticosterone treatment in control animals did not produce a significant effect on body weight gain at any day of the measurement (Figure 4, 5, 6, 7). However, stressed animals receiving corticosterone treatment 30 minutes prior to stress (pretreatment) appeared to gain more weight than stressed animals on day 21 (Figure 6). Body weight gain in stressed animals that received corticosterone pretreatment was not significantly different compared with that in control non-stressed animals on day 21(Figure 6, 7). Interestingly, corticosterone treatment immediately following stress (post-treatment) was not able to increase the body weight gain as that in control group during the 21 days observation period. The weight gain of this group of animals was not significantly different from the stressed animals (Figure 5, 6, 7 $p>0.05$ at all time points).

The stressed animals receiving corticosterone pretreatment appeared to gain less weight (Figure 4), but finally caught up to the body weight of control animals after approximately three weeks following cessation of stressful episodes, indicating that once stress ended, these animals had a higher growth rate than the stressed

groups of animals that did not receive corticosterone pretreatment. Although these animals experienced almost zero growth rate during three days of stressful episodes, they appeared to resume growth at a rate similar to the controls once stress ended (Figure 6, 7). Corticosterone had no significant effect on average growth rate in control animals measured at any time interval. Post-hoc analysis revealed that, in stressed animals, corticosterone pretreatment induced a significantly higher growth rate (Figure 7). It was noteworthy that post-treatment with corticosterone could not enhance the growth rate as pretreatment did during the period following stress, consistent with the previous result that this treatment had no significant effect on the weight gain.

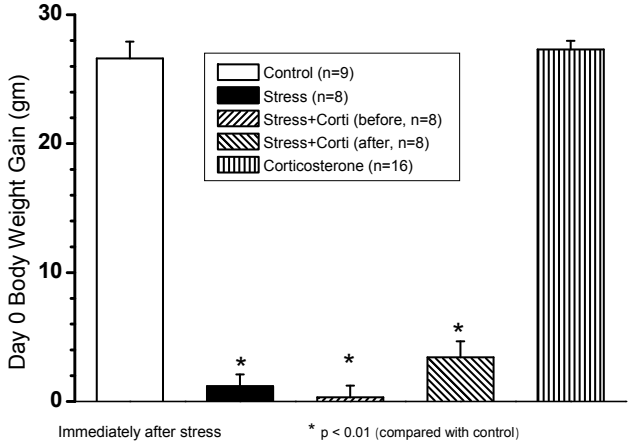

Figure 4. The effect of corticosterone on the body weight gain during the three days stress. Means ± S.E.M of net weight gains of all groups of Sprague–Dawley rats in three days of restraint-tail shock (measured immediately following stress). Corticosterone 3 mg/kg pre- and post-treatment and corticosterone alone, stressed and non-stressed controls respectively.

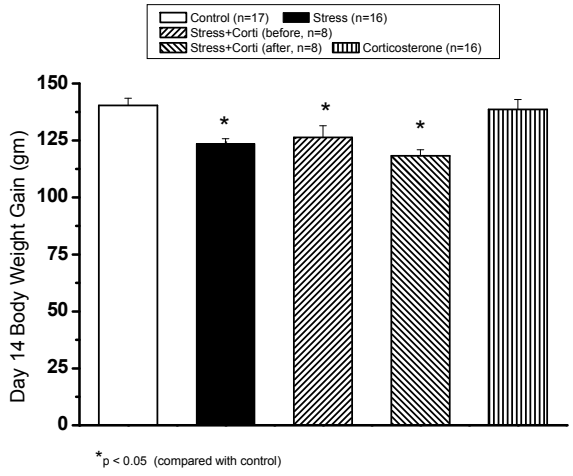

Figure 5. The effect of corticosterone on the body weight gain 14 days after three days stress. Means ± S.E.M of net weight gains of all groups of Sprague–Dawley rats in three days of restraint-tail shock (measured 14 days following stress). Corticosterone 3 mg/kg pre- and post-treatment and corticosterone alone, stressed and non-stressed controls respectively.

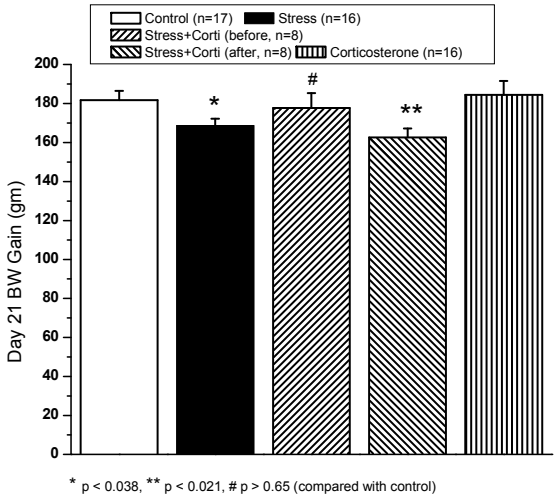

Figure 6. The effect of corticosterone on the body weight gain 21 days after three days stress. Means ± S.E.M of net weight gains of all groups of Sprague–Dawley rats in three days of restraint-tail shock (measured 21 days after stress paradigm). Corticosterone 3 mg/kg pre- and post-treatment and corticosterone alone, stressed and non-stressed controls respectively.

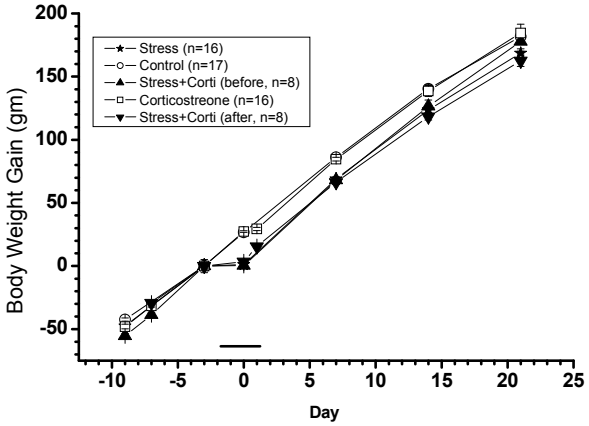

Figure 7. The effect of corticosterone on the body weight gain. Means ± S.E.M of net weight gains of all groups of Sprague–Dawley rats after three days of restraint-tail shock (measured immediately following stress). Corticosterone,3 mg/kg pre- and post-treatment respectively. Mean of net weight gains in different groups of rats was plotted against the days following stress. Body weight was measured on the day immediately following stress and on Day 1, 7, 14 and 21 following stress. Note that the stressed groups with corticosterone pretreatment finally caught up to the control groups in body weight gain.

Task 5: Administration of the glucocorticoid receptor antagonist RU 486 withcorticosterone pre- or post-stress to determine whether the antagonist can block the action of corticosterone as measured by ASR

RU 486 was administered with corticosterone pre-stress to one group of animals, but the data are inconclusive as RU 486 can only be dissolved in a 70 % ethanol solution at the desired concentration. This results in the necessity to inject a relatively large volume of an ethanolic solution in order to administer the dosage reported in the literature of 30 mg/kg. Other solvents such as DMSO have a similar solubilization issue. The current solution to this issue is to split one injection into two injections with a 12 hour interval between injections. Our current results indicate this modification will resolve this issue and it will be applied in subsequent experiments.

Task 6: Determination of plasma corticosterone levels in frozen plasma samples from tail blood using a RIA kin and ICN protocol.We have analyzed approximately 40 frozen plasma samples using a DSL Rat Corticosterone EIA kit. We have found this method to be sensitive enough for our needs without generating radioactive waste that would result from using the RIA method originally proposed. Plasma samples are collected and frozen until there are enough samples to use up a whole kit. The corticosterone levels are significantly lower (200 ng/ml) in the stressed group as compared to the non-stressed control group at Day 28 after stress. This data confirms that the data collected from the current animal model is similar to that reported in the PTSD patients (Golier et al. 1175-78) .

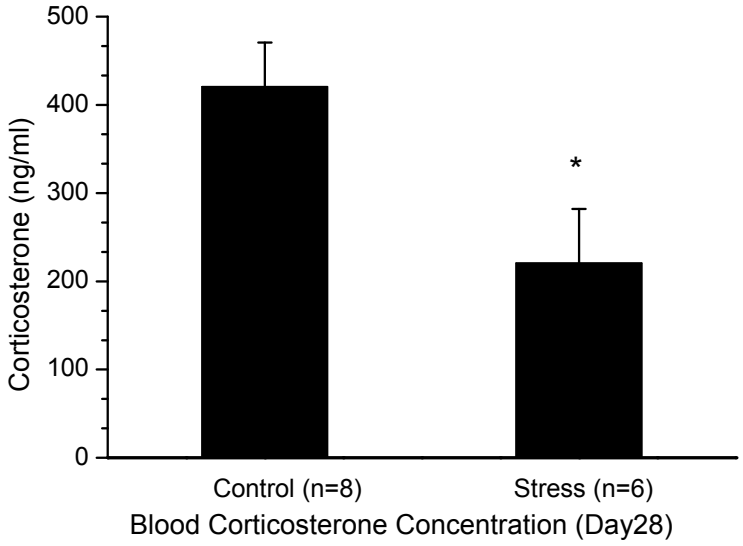

Figure 7. Plasma corticosterone levels in control and stressed blood sample 28 days after stress.

Task 7: Data analysis and preparation of manuscripts for publication

Data analysis is ongoing. Two posters detailing the experiments completed thus far in this project have been presented: One at the 4th Annual Conference on the Neurobiology of the Amygdala and Stress, presented at USUHS on April 28, 2009, and one at USUHS Research Week early in May, 2009. Two abstracts have been submitted, one to the Military Health Research Forum, 2009, to be held in Kansas City, MO August 31-September 3, 2009, and one to the Society for Neuroscience Annual Meeting on October 17, 2009 in Chicago, IL.

KEY RESEARCH ACCOMPLISHMENTS May 1, 2008 through Nov.30, 2009
Preliminary results suggest the following research accomplishments:

- Corticosterone pretreatment (pre-stress) has a beneficial effect in reducing the enhanced fear response that results from exposure to the stress protocol.
- Administration of corticosterone reveals a delayed long term (three weeks) beneficial effect in normalizing the body weigh gain and is without effect on short term body weigh gain immediately after the cessation of stress protocol.
- Bolus administration of corticosterone has no significant impact on the basal fear response and body weight gain.
- Animals appear to have less tolerance for the glucocorticoid receptor antagonist RU 486. This may be due to the high concentrations of RU 486. Attempts are being made to reduce the dosage of RU 486 in subsequent proposed experiments.

REPORTABLE OUTCOMES

1. Oral and poster presentation at the Military Health Research Forum, August 31-September 3, 2009 in Kansas City, MO.
2. Submission of abstract for presentation at the Society for Neuroscience Annual Meeting, October 17, 2009 in Chicago, IL.
3. Poster presented at the 4th Annual conference on Neurobiology of Amygdala and Stress, April 28, 2009, at the Uniformed Services University of the Health Sciences, Bethesda, MD.
4. Poster presentation at Research Week, May 11-13, 2009, at the Uniformed Services University of the Health Sciences, Bethesda, MD.

CONCLUSION

Therapeutic administration of corticosterone appears to have effects in attenuating the fear response in subjects who encounter traumatic stress. Thus, effective pharmacological intervention for PTSD requires protection of both the hormonal and the neuronal systems associated with the stress response from impairment that can initiate the pathophysiology of traumatic stress. At this time, we conclude: 1) Rats subjected to restraint/tail shock stress for three days developed an exaggerated acoustic startle response 14 days after stress. This was associated with cessation of body weight gain during the three day period of stress and body weight gain remained reduced compared with that of non-stressed control rats. 2) Administration of the glucocorticoid receptor agonist corticosterone (3 mg/kg) 30 minutes before stress attenuated the exaggerated acoustic startle response to stress and normalized body weight gain resulting from stress. 3) Administration of the glucocorticoid receptor agonist corticosterone (0.3 mg/kg) 30 minutes before stress did not attenuate the exaggerated acoustic startle response to stress and has not significant impact on body weight gain resulting from stress. 4) The serum concentration of corticosterone is significantly lower in stressed rats than in non-stressed controls 28 days after stress.

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